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Ankyrin repeats are motifs of thirty three amino acid residues, which are found in over 100 proteins. Several of these are of particular interest because they suggest roles for the ankyrin repeat in tumor development. ANK repeats were first identified in a pair of yeast transcription factors (Swi4 and Swi6), and this research is focused upon understanding the role of the ANK repeats within these proteins. We have generated libraries of PCR-generated mutants of the Swi6 ANK domain and analyzed them with respect their transcriptional activity and sequence changes. We also used site-directed mutagenesis to systematically remove the core residues from each of the 4 ANK repeats, and found that the ANK core mutants as well as many of the random mutants are temperature sensitive for activity. This suggests that this region of the Swi6 protein is critical for the stability of the protein. We then isolated high copy suppressors of these mutants. Two gene products known to be involved in transcription and DNA interaction were identified (NHP6A and MSN1) as well as a novel protein (PIB1). The significance of these interactions is under investigation

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Linda Breeden _July 29, 1996_
PI - Signature   Date

The Role of the ankyrin repeats in the Swi4/Swi6

transcription complex of budding yeast.

Annual report 1996

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INTRODUCTION

Ankyrin repeats are motifs of thirty three amino acid residues with the consensus sequence:

$$-N-D-G-TPLH-AA--G---VV--LL--GA--$$

The entire ankyrin repeat sequence is degenerate, such that no specific residue is conserved throughout all known ankyrin-repeat containing proteins. What defines the ankyrin repeat is the presence of a hydrophobic "core" (italicized, above) flanked by charged residues (bold type, above). This series of amino acids is the most highly conserved region of the ankyrin repeat, and typically identifies a repeat as "consensus"; its absence denotes a "degenerate" repeat. Typically, the repeats occur as domains of four to seven contiguous copies of the motif, with consensus and degenerate repeat sequences interspersed.

Over 100 proteins have been identified as containing ankyrin repeats. Several proteins have received particular interest because of potential roles for the ankyrin repeat motifs in tumor development. These include Bcl-3, int-3, TAN-1, and p16. The BCL-3 gene encodes a member of the I kappa B family of proteins and rearrangements involving the ankyrin repeat region BCL-3 have been identified in B-cell chronic lymphocytic leukemias (11). Int-3, a mouse proto-oncogene, is a common insertion site for the Mouse Mammary Tumor Virus (MMTV). Activation of the int-3 ankyrin repeat sequences by MMTV produces poorly differentiated adenocarcinoma of the mammary and salivary glands in mice (8). TAN-1, the human Notch homologue, was first described as a breakpoint region of t (7,9) gene rearrangements found in T-cell acute lymphoblastic leukemias. This rearrangement places the gene encoding the beta T-cell receptor adjacent to the ankyrin repeat motifs of TAN-1 (4). p16 is the protein product of the MTS1 (multiple tumor suppressor-1) gene. It is composed of only a series of four ankyrin repeats and is one member of the cyclin dependent kinase inhibitor family of proteins. p16 can compete with cyclin D and displace the cyclin subunit from its kinase binding site, thereby inhibiting G1 to S progression through the cell cycle. Mutations in p16 have been noted in a variety of human tumors and tumor cell lines, including bladder and gastrointestinal neoplasms and mesotheliomas; in addition, mutations have been found in the germline of families predisposed to melanoma (7).

While ankyrin repeats are presumed to have a role in protein-protein interaction, the actual function of the motifs is unknown. Ankyrin repeats were originally described by Breeden and Nasmyth (1) in the Swi6 transcription activator of the budding yeast, Saccharomyces cerevisiae. Swi6, with its two known protein binding partners, Swi4 and Mbp1, regulates gene expression during the G1 to S transition of the yeast cell cycle. The Swi6/Swi4 heterodimeric complex binds to SCB (Swi4/6 cell cycle box: CACGAAA) elements present in the promoters of HO and the G1 cyclins and the Swi6/Mbp1 complex binds to MCB (MluI cell cycle box: ACGCGTNA) elements in the promoters of DNA synthesis genes in late G1.

Previous evidence suggested that the ankyrin repeat region of Swi6 was necessary for binding of the Swi6/Swi4 complex to DNA and not for the association of Swi6 with Swi4 (14). One hypothesis, therefore, is that the Swi6 ankyrin repeat region recruits additional proteins to the Swi6/Swi4 complex which are required formation of the DNA binding complex. With this

in mind, we have focused our initial efforts upon genetic strategies to identify ANK repeat-interacting proteins.

RESULTS

Our purpose has been to define the function of the ANK repeats in Swi4 and Swi6. This involved exhaustively mutagenizing the ANK repeats of Swi4 and Swi6 to identify the critical residues within each repeat. The effects of these mutations upon DNA binding and activation of SCB- and MCB-mediated transcription has been determined. Partially defective mutants have been identified and used to search for interacting gene products using suppressor analysis.

Our Technical Objectives are to:

1. Generate monoclonal and polyclonal antibodies that recognize either Swi4, Swi6 or all ANK repeats and use them to screen for other ANK repeat-containing proteins.

2. Exhaustively mutagenize the ANK repeats of Swi4 and Swi6, and identify the crucial residues for their activity.

3. Develop a battery of genetic screens to identify mutations that cause loss or deregulation of known Swi4 and Swi6 functions.

4. Perform *in vitro* assays to correlate mutant phenotypes with known biochemical functions.

5. Identify second site suppressors, either within the Swi protein, or within associated proteins.

We have completed a series of experiments regarding objectives 2 and 5 and are in the process of writing up this work for publication. This work is summarized below. Some of our efforts with regard to task 4 are also described.

TASK2. Define critical amino acids involved in ANK repeat function.

Random mutagenesis. We have constructed a total of four libraries of SWI6 genes with randomly mutagenised ANK repeat regions. We have isolated about 30 different temperature sensitive (ts) and 110 unconditionally inactive (null) Swi6 ANK mutants. Since most of the null mutants appeared to have lost expression of Swi6, we concentrated our efforts on ts mutations, the majority of which expressed Swi6. Some of the temperature sensitive mutants are listed in Appendix 1. The panel of mutations in Fig 1 demonstrates that mutations occur throughout the region and are about as likely to occur in the region of second and third repeats as elsewhere. Above the amino acid sequence, we present the results of computer analysis of the ANK repeat sequence. The gray boxes are regions that are very likely to exist as alpha helices, and the black

box represents the region of ANK repeats that may form a beta sheet. Thus most of the repeat region may have an alpha helical secondary structure with the exception of a middle part of the spacer and the core region of the third repeat. It is immediately clear from the distribution of ts mutations that they are found throughout the region. There is some clustering at each end of the whole ANK domain (although this could be a PCR artifact). The only region lacking mutations is the non-structured part of the spacer.

We identified several residues that were targeted in two independently mutagenised libraries (marked by ellipse in Appendix 1). We have also identified five critical residues: R344, G347, D375, A477 and N500 (circled), whose substitution is enough to compromise the activity of the whole region. Most if not all of the mutants that we looked at, were defective both in SCB driven transcription and in MCB driven. To obtain this data, we have compared lacZ levels produced from a set of reporter plasmids in yeast strains with different ts ANK mutations at nonpermissive temperature. The four reporter constructs we used had as a UAS (upstream activating sequences) in their promoters 1) a fragment of HO promoter, 2) a fragment of TMP1 promoter, 3) a synthetic trimer of SCB elements and 4) a synthetic trimer of MCB elements (TMP1 and HO fragments contained activating sequences that have SCB and MCB elements, respectively, in the native context). There was some variation between the different ts mutants as to the degree of defects in SCB versus MCB transcription. For example, b26 showed high activity in HO transcription but low in 3xMCB transcription. However, it was clear, that in no case one function was affected and the other remained intact. Therefore, our results obtained so far suggest that the two functions are not separable within the ANK repeat region.

We have also performed a screen for mutations that will hyperactivate the ANK repeat. We used one of the libraries that were most heavily mutagenised. No ANK mutations with hyperactivating characteristics were found. However, on two occasions we identified rare recombination products that resulted in duplication of the HIS3 gene itself, which led to increased HIS3 expression from an MCB:HIS3 reporter. This suggested to us that our screening strategy (for elevated HIS3 expression using the growth advantage on aminotriazole) had worked. It is therefore likely that a hyperactivating mutation cannot occur in ANK region, alternatively, that such mutation requires more than a couple amino acid substitutions and therefore cannot be generated by point mutagenesis.

Site-directed mutagenesis. Surprisingly few of the mutations generated randomly were within the conserved core of the ANK repeats. To determine the extent to which the cores were contributing to Swi6 function, we performed site-directed mutagenesis of the core regions of each repeat, both singly and in combination. Appendix 2 shows the results of this work. In each case the G-T-L core residues of the repeat were changed to alanines. We found that these mutants varied in function at 25 degrees, but all of these mutations conferred a temperature sensitive phenotype to Swi6. This indicates that the entire length of the ANK domain is critical for the stability of the Swi6 protein. Mutations in the first repeat had the least effect, then the second, third and the fourth increased in importance. The multiple mutants presented a more complex picture, in that many of the multiple mutants had more activity than the single fourth repeat mutant. The most surprising of these is the Swi6 mutant with all four repeats mutated. In this case, acitivty was much higher, about half that of wild type. This remains a mystery. Our working hypothesis is that the repeats are involved in some form of redundant negative control,

such that mutation in a subset of the repeats doesn't disrupt it, but mutation of all four repeats does.

TASK5. A screen for high copy suppressors of ANK ts mutations.

We have performed a high copy suppressor screen of ts ANK mutations with the mutants b19, b38 and b8 (see Appendix 1). 7 and 19 suppressor plasmids were recovered from b38 and b19, respectively, and among those there were 12 different types of plasmid, as judged by their restriction digest patterns. The screen performed for b8 has not yielded any new suppressors that were not recovered for b38 or b19. A total of 4 suppressors activated ho::LacZ equally strongly in swi6Δ cells and in swi6 ANK mutant cells. We conclude that they carry complete bypassers of the Swi6 function, or alternatively, they may encode SWI6 itself. We focused our attention only on those suppressors that were unable to activate ho::LacZ in the absence of Swi6 in swi6Δ strain, or that were less efficient in activating ho::LacZ transcription in the absence of Swi6 than in the presence of the temperature sensitive ANK mutant Swi6. Most of these suppressors (7 out of 8) were able to activate ho::LacZ expression to some extent without Swi6 and thus are partial bypassers of Swi6 function. Out of these seven, only one was able to activate ho::LacZ in the absence of Swi4. All the rest of the suppressors (#11, #12, #2, #5, #9, #23) required Swi4, which indicated that they restore Ho::LacZ transcription in a promoter-specific manner, rather than deregulate it in some general fashion. Finally we obtained a single suppressor (#15) that was unable to activate any ho::LacZ transcription in the absence of Swi6. #15 was also unable to suppress certain severe mutations in SWI6 outside of the ANK repeat region. However, this suppressor was not specific to a particular ANK ts mutation, it was able to suppress most of the ANK ts mutations tested, but b8, which was the most defective out of the set tested.

We obtained some sequence information for all of the suppressors and have searched genomic databases with these sequences to obtain full maps of the DNA inserts. In each case more than one open reading frame was present on the insert. We have subcloned insert fragments of #15 and #11 to determine which of the open reading frames were encoding suppressors. The results of mapping and subcloning showed that previously cloned genes, MSN1 and NHP6A were responsible for suppression phenotype of #11 and #15, respectively. At the same time we determined that suppressers #12, #5 and #9 carry fragments, which are substantially overlapping with that of #11, and which all bear MSN1 open reading frame (from chromosome XV). #23 appears to carry a different fragment of chromosome XV and #2 carries a fragment from chromosome XIII. The open reading frames present on these fragments have not been characterized previously. We have determined which open reading frame of #2 is responsible for suppression. It is a new gene of unknown function (ORF YM8520.13c) that we called PIB1 (Partially Independent Bypasser of Swi6). PIB1 has no close homologues in yeast or in higher eukaryotes, however it resembles several transcription factors. Interestingly, it bears some structural similarity to a Drosophila transcription factor Dorsal, which is known to interact with the ANK repeat containing regulator Cactus (9). We have disrupted PIB1 and found that it is not essential for yeast viability. Double mutants with disruption of PIB1 and either SWI4 or SWI6 are also viable. We plan to continue the analysis of PIB1 in the future.

MSN1 has been originally cloned as a high copy suppressor of a temperature sensitive SNF1 kinase mutant (5). It has activator properties and has an extremely weak and nonspecific DNA binding activity. MSN1 has been also selected by other groups as FUP1(3) and PHD2, an

activator of pseudohyphal growth (6) as well as a protein capable of activating mating specific genes when overproduced (13). Therefore, it is likely that MSN1 is a non-DNA binding transcription activator, or coactivator, that is utilized by several unrelated promoters. Strains deleted for MSN1 grow slower than the wild type suggesting that MSN1 is involved in the regulation of genes important for viability. We found that in msn1 deletion strain the transcription of HO is reduced to 20-30% of the wild type. Thus, MSN1 is required for maximal expression of HO.

NHP6A has also been identified before (2,10) and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of looping DNA (12). NHP6A has a close homologue in yeast, NHP6B, which encodes a similar protein with potentially overlapping activity (2). Strains with deletions in both NHP6A and NHP6B have multiple phenotypes, including dramatically reduced growth rates. Both NHP6 proteins have long been implicated in facilitating gene expression by inducing favorable chromatin conformations on the promoters, however, there is no direct evidence available. We found that NHP6A-6B deletion strains have reduced levels of HO and CLN1 transcription.

TASK 4. Biochemical characterization of the ANK repeat SWI6 mutants and potential ANK interacting proteins with regard to their DNA binding activities.

We have analyzed several ts ANK mutants in DNA binding assays with fragments of HO or TMP1 promoters. Our results suggest that, thus far, all mutants have defects in DNA binding, both to SCB and to MCB elements. With the TMP1 promoter, the mutants tested all have reduced DNA binding efficiency. However, with the HO promoter, the observed defects fall into two groups. Mutants b19, b28, b8 have a reduced amount of the Swi4/Swi6 complex bound to the promoter fragment. Mutants b38, b26 and possibly b37, which are no less defective in SCB transcription than the former group, produce a Swi6-containing complex of altered mobility. This complex is equally abundant as the wild type Swi4/Swi6 complex, however, it migrates significantly higher. This data is the first demonstration that mutations in Swi6 ANK repeats can result in an altered conformation and/or composition of the complex, that may potentially render it inactive. We are currently developing assays to address this effect in more detail and testing other ANK mutations for their DNA binding activity.

We have generated fusions of Msn1 and Nhp6a proteins with Gst and purified these proteins from E. coli and yeast. We also obtained an Nhp6a protein tagged with HA tag from M. Snyder. In accordance with published results, we observe that Nhp6a purified from yeast or E. coli is capable of strong but nonspecific binding to DNA. Neither Msn1 nor Nhp6a associate with Swi6 in yeast extracts or in purified state.

Using the gel retardation assay we have demonstrated that Swi4/Swi6 complex formation is intact in msn1 deletion strain. From this and the fact that Msn1 does not appear to associate with Swi6, we conclude that Msn1 exerts its function not through the Swi4/Swi6 complex, but through other components of the transcription machinery. On the contrary, Swi4/Swi6 complex formation is reduced in the nhp6ab deletion strains. Therefore Nhp6a may be able to facilitate formation of the Swi4/Swi6-DNA complex. However, we have not been able so far to demonstrate that Nhp6a protein is physically present in this complex on DNA and/or associates either with Swi6 or with Swi4.

CONCLUSIONS

Mutations that cause loss of Swi6 function at high temperature are found throughout the ANK domain. There is some clustering of mutations at each end of the ANK domain, but each ANK repeat is important. The only region lacking mutations is the non-structured part of the spacer.

We have identified some of the critical residues: R344, G347, D375, A477 and N500, whose substitution is enough to compromise the activity of the whole region. Most if not all of the mutants that we looked at were defective both in SCB- and MCB-driven transcription, indicating that **the two activities are not separable by mutation in the ANK domain.**

Hyperactivating mutations cannot occur in ANK region, or such a phenotype requires more than a couple of amino acid substitutions and therefore cannot be generated by this mutagenesis procedure.

Site-directed mutations within the conserved cores of the ANK repeats all confer a temperature sensitive phenotype to Swi6. **This indicates that the entire length of the ANK domain is critical for the stability of the Swi6 protein.** Mutations in the first repeat had the least effect, then the second, third and the fourth increased in importance. Multiple repeat mutants suggest the possibiltiy that the repeats are critical for some form of negative control over Swi6 activity.

We have identified three genes that can suppress ts ANK defects of SWI6. PIB1 is a new gene, with homology to some other transcription factors, but no known function. The other two: NHP6A and MSN1, are previously identified genes. We have established that two of these genes are required for maximal HO expression. **NHP6A facilitates Swi4/Swi6 complex formation on DNA,** but we have no evidence so far of direct interaction between Swi6 and any of these proteins.

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APPENDIX 1

The amino acid sequence of the ANK region of Swi6 is shown. The regions of the highest conservation between the repeats of Swi6 and other proteins of the Swi4/ Swi6 family (so called core regions) are boxed. There are four and a half such regions in Swi6. The regions of lower, but still noticeable conservation are shown in bold. Variable regions are in plain style. A long variable region (spacer) between the cores 3 and 4 is the unique feature of Swi6. The four bold-lettered regions with the boxed cores in their N-terminal half represent what is now defined as an ANK repeat unit, thus there are 4 ANK repeats found in Swi6. The gray boxes are regions that are very likely to exist as alpha helices, and the black box represents the region of ANK repeats that may form a beta sheet, based on secondary structure predictions. The ovals represent mutations there were obtained from more than one mutagenesis. The circled residues are those that are critical for function, in that a single substitution at that position dramatically reduces Swi6 function.

Row labels (top to bottom): 1, 2, 3, 4

Sample labels (left column):
T4
CE3
KWA11
KWA9
KWA8
KWA2
KWA1
A1
B26
B8
Wh6
B37
B38
B19
B28
4-10
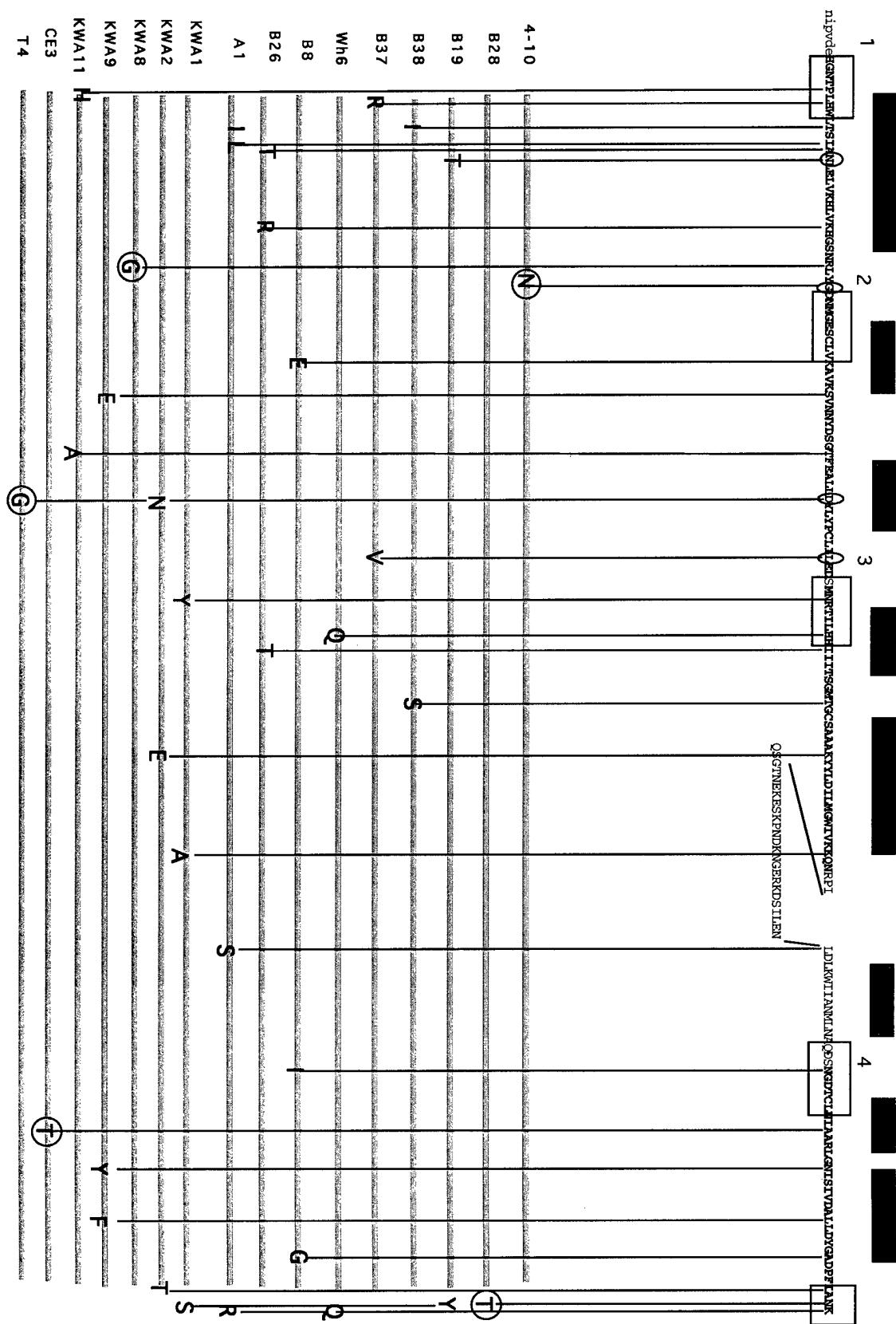
APPENDIX 2

This diagram depicts the positon (s) of the alanine substituted cores (▐▐▐) in the various *swi6* mutant constructs. The alanine substitution was performed by site-directed mutagenesis, with the resulting mutations confirmed by sequencing and restriction digests. The mutation was integrated into the *LEU2*+ chromosomal locus of a cell deleted for *SWI6*, resulting in growth on selective plates. The presence of the integrated ankyrin repeat mutant sequence was confirmed by polymerase chain reaction. The blue-white plate assay, using SCB driven *ho::LacZ* as a reporter, conveys a temperature-sensitive phenotype for each of the mutants when the cells are grown at 37 degrees. These results suggest a significant role for each of the four Swi6 ankyrin repeats in SCB activated transcription by the Swi6 complex.

	Denotes alanine substitution in core region		Blue-White plate assay	
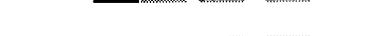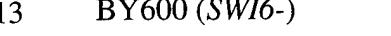
construct number		25°	37°
1		blue	blue
2		blue	white
3		pale blue	white
4		pale pale blue	white
5		white	white
6		pale blue	white
7		pale pale blue	white
8		pale blue	white
9		blue	white
10		pale blue	white
11		pale blue	white
12		blue	pale blue
13	BY600 (*SWI6-*)	white	white